UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, DC 20460

OFFICE OF
CHEMICAL SAFETY AND
POLLUTION PREVENTION

MEMORANDUM

Date: October 30, 2017

Subject: Protocol Review for sanitization of alfalfa seeds prior to sprouting using PUMA (Reg. # 5813-100)
EPA File Symbol 5813PA1 (DP# 442402)
E-submission # 21674

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Applicant: The Clorox Company
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FORMULATION FROM THE LABEL:

Active Ingredient	% by wt.
Sodium Hypochlorite	8.25%
Other Ingredients	91.75%
Total	100.00%

I BACKGROUND

The applicant submitted an efficacy protocol for sanitization of alfalfa seeds prior to sprouting using sodium hypochlorite (PUMA Reg. # 5813-100). The product, PUMA, is a sanitizer for both food contact and non-food contact surfaces, a soft surface sanitizer and a disinfectant for hard non-porous surfaces. According to the cover letter dated August 11, 2017 the registrant previously corresponded with EPA and FDA on the protocol development and identified points of contact at both agencies. NOTE: EPA met with Clorox on 08/25/2016 and 04/26/2017 to discuss development of the protocol. The use is needed as part of a FSMA requirement for treatment of seeds for sprouting.

This data package contained a cover letter dated August 11, 2017, EPA Form 8570-1 (Application for Pesticide), a protocol for alfalfa seed sanitization prior to sprouting using sodium hypochlorite, a non-

GLP study entitled "Evaluation of the antimicrobial efficacy of sodium hypochlorite for alfalfa seed sanitization" (MRID 50448201) and a sample label

II PROPOSED USE DIRECTIONS

To treat alfalfa seeds against Escherichia coli O157:H7 [(E. coli)] -and/or- Salmonella spp. [(Salmonella)], dilute to 19,000 ppm by adding 1-part sodium hypochlorite to 4 parts of water. Use chlorine test strips to quantify the available chlorine. If the available chlorine is less than desired, add a small amount of product slowly and carefully to the dilution and determine the available chlorine with a fresh chlorine test strip. Repeat these steps, as needed, until the desired concentration of chlorine is achieved. Let stand [for] 20 min[utes].

III AGENCY STANDARDS FOR THE PROPOSED CLAIMS

No agency efficacy standards apply to this use.

IV PROPOSED EFFICACY PROTOCOL

Objective

The Clorox Company has been asked to register a disinfectant to meet the sprouts industry's need for an approved seed treatment method as required by FSMA regulations beginning in 2017. The goal of the following protocol is to provide an effective seed treatment method to be adopted by the sprouts industry. This method focuses on seed treatment prior to the sprouting process to achieve a ≥3 log reduction of bacteria of public health significance using Clorox liquid bleach as a sanitization agent.

Scope

The following protocol provides a scientifically valid method that can be used to treat seeds immediately prior to sprouting at a manufacturing facility to meet FDA CFR requirement §112.142. This fulfilment requires ensuring that the treatment procedure is correctly followed including, as appropriate:

- treatment active level
- appropriate volume of solution to seed
- treatment contact time
- treatment temperature
- other measures specific to the manufacturer's operation that may pose foreseeable hazards to the efficacy of the seed treatment process

Seed Treatment Procedure- Overview

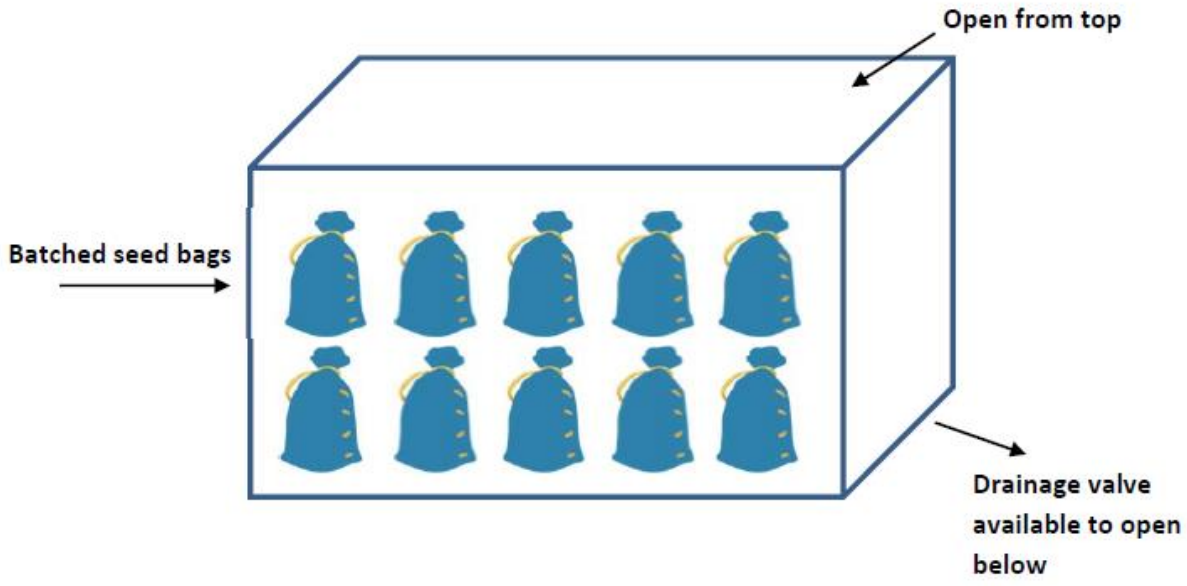
1. *Pre-germination Seed Soak*

Soaking results in swelling and softening of the seed coat, promoting the sprouting process. Pre-germination seed soaking is not required for this treatment process. If seed soaking practices are used, it is recommended that all materials are cleaned and sanitized, in addition to ensuring that the water used meets the microbial quality criterion in § 112.44(a).

2. *Seed Batching*

Seed should be batched in similar weight portions using suitable materials such as mesh bags, bins or trays (following proper cleaning and sanitizing practices). The batching material chosen by the sprouting manufacturer should ensure that proper fluid access to and drainage from the seed batches is sufficient.

The following schematic provides an example of seed batching using mesh food bags contained within large bin to carry out the process:



3. *Preparation of Treatment Solution*
Clorox liquid bleach should be prepared to a final concentration level of 19,000 ppm in a volume of water sufficient to fully submerge all batched seed. It is recommended that the actual available chlorine level is obtained by titration just before the treatment procedure.

4. *Seed Treatment and Post-rise*
Seed batches should be immersed into the treatment solution and mixed throughout the contact time (20 minutes) either by manual "dunking" of the mesh seed bags or rotating the bins/trays. At the end of the contact time, seeds should be rinsed with pure water. The rinse step is to be carried out immediately after the treatment step, with the seeds batched and fully immersed in a volume water sufficient to completely submerge the seed. These steps should be carried at room temperature.

5. *Quality Control Measures*
It is recommended that the sprouting facility take appropriate measures to ensure that the seed treatment process is carried out as described and using all materials that meet the microbial quality criterion (i.e. water quality). Additionally, the final concentration of the available chorine level and analysis of rinse water from the treatment step should be routinely carried out to ensure the treatment process is effective.

6. Satisfaction of criteria for a valid test and calculations
The Clorox Company has been approached to develop an effective test method and register a disinfectant to meet the sprouts industry's need for an approved seed treatment method, as required by the FDA Produce Safety Rule issued in November 2016. The goal of the following study was to provide data to support the development of an effective seed treatment method and show the efficacy of an appropriate disinfectant to be adopted by the sprouts industry. Success is defined as achieving a 3-5 log reduction of bacteria of public health significance

using a pathogen challenge study with Puma as a disinfecting agent. This method focuses on seed treatment after receipt from suppliers and immediately prior to the sprouting process.

Protocol

Study Materials

- Non-scarified alfalfa seeds both untreated by supplier and pre-treated by supplier
- Media for *E. coli* O157:H7: MacConkey Agar with Sorbitol and 0.1% Pyruvate (SMAC)
- Media for *Salmonella spp*: Xylose Lysine Deoxycholate Agar with 0.1% Pyruvate (XLDP)

Challenge organisms

Table 1: Bacterial Strains Used in the study

Pathogen	Outbreak	Year
E. coli O157:H7	Spinach	Unknown
E. coli O157:H7	Salami	2005
E. coli O157:H7	Unknown	Unknown
S. cubana	Unknown	Unknown
S. heidelberg #1	Chicken	2015
S. heidelberg #2 (Foster Farms antibiotic resistant strain)	Chicken	2015
S. seftenberg	Pistachio	2015
S. enteritidis phage type 30 (PT30) *Extremely heat resistant, tested separately.	Almond	2003

Method

a) Preparation of Bacterial Inoculum:

i) Test seeds are inoculated with pathogenic isolates of *Escherichia coli* O157:H7, *Salmonella* spp. and *S. enteritidis* PT30 (**Table 1**) prepared as cocktails for each organism type (total of 3 "cocktails"). All isolates have been implicated in food/produce outbreaks (as indicated in Table 1) and are most appropriate for use in a foods study. These pathogenic strains were revived from frozen cell cultures maintained by CMC, Inc. and the Center for Food Safety, University of Georgia.

ii) Pure cultures of each individual strain are grown in 10ml Tryptic Soy Broth (TSB) at 36 ± 1ºC for 24 h, sub-cultured by taking 1ml of this culture and adding to 9ml of fresh TSB, then grown for an additional 24 h at 36 ± 1ºC. The growth culture will undergo a second subculture following the same procedure described previously for a total of 2 transfers.

iii) 1ml of each individual strain culture is then spread on Tryptic Soy Agar (TSA) plates and then grown for a bacterial lawn for 24 h at 36 ± 1ºC.

iv) Cocktails for each organism type are prepared by flooding each bacterial lawn plate with 5 ml of PBS to harvest bacterial cells. The suspensions are pooled with the appropriate strains necessary to comprise each cocktail. Bacterial cells were not washed before pooling into a single suspension as to allow any soil present to remain, mimicking a real-world scenario.

v) Suspension counts of each cocktail are determined using serial dilutions and subsequent enumeration by plating on the appropriate selective media after incubation at 36± 1°C for 24-48 hours.

b) Inoculation of Seed:

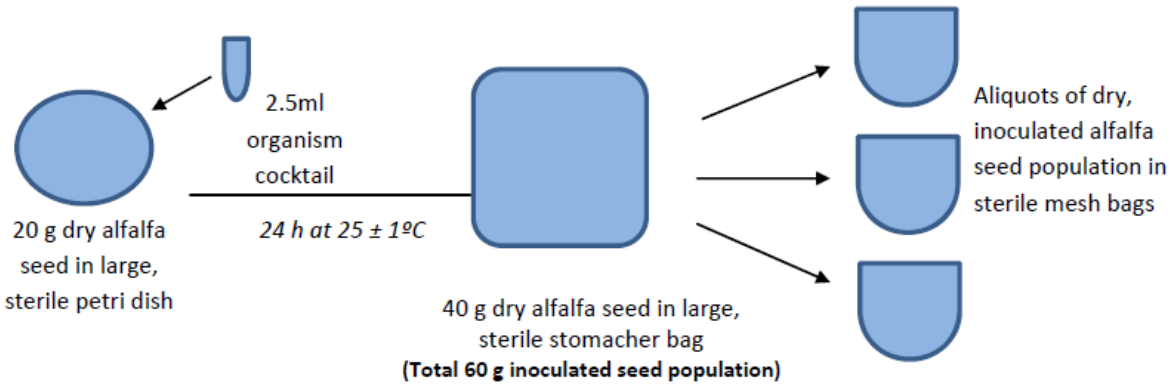
i) Water activity of dry, uninoculated seed is measured and noted in the final report.

ii) Seeds are batched into 20 g aliquots in large sterile glass petri dishes. Each dish is inoculated with 2.5 ml of pathogen cocktail suspension.

iii) The seeds are mixed manually with a sterile spreader and turntable for 2 min. at room temperature in each dish for even distribution of the inoculum, and then spread in a thin layer inside the glass petri dish for drying in a laminar flow hood at room temperature with the lid half open (approximately 24 hours).

iv) Water activity of dry, inoculated seed is measured at the end of the 24 h period to ensure the water activity is comparable to dry, uninoculated seed and noted in the final report.

v) Inoculated seeds are then mixed with uninoculated seed at a ratio of 1:3 by manual manipulation immediately prior to testing in large sterile stomacher bags (i.e. the 20 g aliquot of inoculated seed is added to 40 g of uninoculated seed for a total seed sample of 60 g). From this point forward, “inoculated seeds” refers to this mixed population of seeds.



c. Initial Numbers Controls:

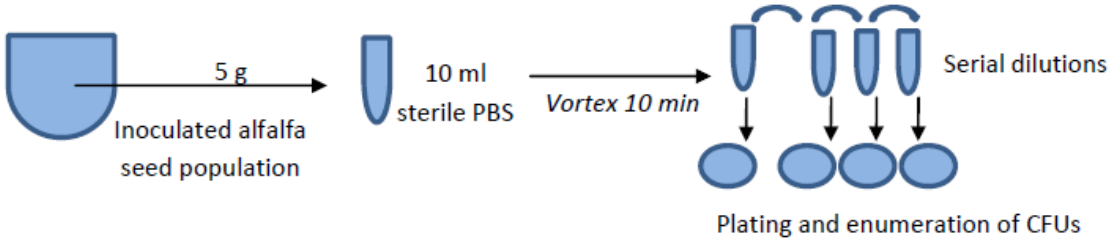
i) Before treatment, each seed bag is shaken by hand for 30 seconds and then three 5 g samples of seeds are pulled from each group and transferred to 10 ml of PBS in a 50 ml conical tube

ii) Samples are mixed via vortex for 10 minutes.

iii) Serial dilutions are performed in PBS and the dilutions are plated in duplicate using the appropriate selective growth medium.

iv) Plates are incubated 36 ± 1°C for 24-48 hours.

v) Enumeration and calculation of surviving CFUs are performed using standard microbiology techniques.



d) Preparation of Product Treatment Solution:

i) Sodium hypochlorite treatment solutions were prepared by dilution of Puma (8.25%, Lot# A81625018CA3) with sterile, de-ionized water to the target level. Both product and diluent stocks are stored at 4°C prior to use.

ii) Each treatment solution is analyzed for pH level and available [OCl-] by titration just before seed treatment. Each treatment solution is analyzed again for pH level and available [OCl-] immediately after the treatment period.

Table 2-Test Groups

Sample Group	Organisms	NaOCl Concentration	Contact Time
Uninoculated Seed A1- Untreated by Supplier			
Uninoculated Seed A2- Pre-treated by Supplier			
B-Inoculated Seed	1) *E. coli O157:H7* strains *2) S. spp.* 3) *S. enteritidis phage type 30*		
C-Inoculated Seed	1) *E. coli O157:H7* strains *2) S. spp.* 3) *S. enteritidis phage type 30*	13,000 ppm	20 min

D-Inoculated Seed	1) *E. coli O157:H7* strains *2) S. spp.* 3) *S. enteritidis phage type 30*	19,000 ppm	20 min
E-Inoculated Seed	1) *E. coli O157:H7* strains *2) S. spp.* 3) *S. enteritidis phage type 30*	25,000 ppm	20 min

e) Seed Treatment:

i) For each sample group (see Table 2), the remaining 45 g of inoculated seeds is divided into three 15 g aliquots and are placed in sterile mesh bags.

ii) Each aliquot of seeds in each group is immersed in 100ml of the appropriate sodium hypochlorite solution while inside the mesh bag and mixed for the designated contact time by manual "dunking" of the mesh seed bags at room temperature.

iii) The treatment step is neutralized by transferring the mesh bags of seeds to 100 ml of 0.2% sodium thiosulfate solution (prepared in sterile water) and mixed by manual "dunking" of the mesh seed bags for 5 minutes at room temperature.

iv) After neutralization, 5 g aliquots of inoculated, treated seed are pulled from each group/replicate and transferred to 10 ml of PBS in a 50 ml conical tube. The remaining 10 g of seeds from each group replicate are transferred to sterile bottles for observation.

v) Samples are processed to assay for bacterial survivors as described above (Section 3, Steps ii-v).

vi) Samples are processed to assay background microflora of untreated and supplier pretreated seeds as described above (Section 3, Steps ii-v).

vii) The starting and remaining hypochlorite in the treatment solution is assayed by titration and noted in the study data.

viii)The starting and final pH of the treatment solution is measured and noted in the study data.

ix) Seeds are watered every 48 hours with 10 ml of sterile water and are allowed to germinate for 7 days following treatment at 25 ± 1°C. The growth and development of the sprouts in each test group observed is noted in the study data.

f. Data analysis

Determination of Log10 Reduction:

Log Reduction= log10 (***X***) - log10 (***Y***)

Where:

X= The number of viable organisms present on seed before treatment (Initial Numbers Controls)

Y= The number of viable organisms present on seed after treatment

e. Study Controls

Media Sterility Control

1. One plate containing the type of growth media used is incubated for 24-48 hours at 36 ± 1ºC to validate media sterility.
2. An aliquot of diluent and neutralizing solution (0.2% sodium thiosulfate) used is plated on non-selective agar media and incubated for 24-48 hours at 36 ± 1ºC to validate media sterility.

Test Culture Purity and Media Growth Control

1. A purity streak of the test microorganism is performed on both selective and nonselective media. This also serves as the enumeration media growth control.
2. Plate(s) are incubated for 24-48 hours at 36 ± 1°C alongside other enumeration and control plates.

f. Proposed Study Acceptance Criteria

1. The media sterility controls must be negative for growth.
2. The media growth controls must be positive for growth.
3. The test system (test microorganism) must demonstrate a pure culture after two passes of 1 ml of growth culture at each pass.
4. Success is defined as achieving at least a 3 log reduction of bacteria of public health significance after treatment.

V SYNOPOSIS OF SUBMITTED EFFICACY STUDY

1.	**MRID**	50448201	**Study Completion Date:**	10/16/2016
Study Objective		Sanitization of alfalfa seeds prior to sprouting using EPA Reg. # 5813-100		
Study Title		Evaluation of the Antimicrobial Efficacy of Sodium Hypochlorite for Alfalfa Seed Sanitization		
Testing Lab, Lab Study ID		The Clorox Company		
Test organism(s) **☐ 1 ☐ 2 ☒ 3 ☐ 4+**		*E. coli* O157:H7 Cocktail Sa*lmonella* spp. Cocktail *S. enteritidis* Phage Type 30 (PT30)		
Test Method		Submitted Protocol for treatment of seeds prior to sprouting		
Application Method		Dunking of mesh bag of seeds		
Test Substance Preparation	**Name/ID**	PUMA Reg. No. 5813-100, F2010.0092		
	Lots **☒ 1 ☐ 2 ☐ 3**	Lot #1: A81625018CA3: 13,000, 19,000 and 25,000 ppm FAC		
	Preparation	Sodium hypochlorite treatment solutions were prepared by dilution of Puma with sterile, de-ionized water to the target level. Both product and diluent stocks are stored at 4°C prior to use. Each treatment solution is analyzed for pH level and available [OCl-] by titration just before seed treatment. Each treatment solution is analyzed again for pH level and available [OCl-] immediately after the treatment period.		
Soil load		n/a		
Carrier type, # per lot		n/a		

Test conditions	**Contact time**	20 min.	**Temp**	n/a	RH	n/a
Neutralizer	100 ml of 0.2% sodium thiosulfate solution					
Reviewer comments (i.e. protocol deviations and amendments, retesting, control failures, neutralizer, etc.)	The study was conducted with a single batch of product and was not conducted under GLP. Controls: Carrier population control, sterility, purity, neutralization confirmation, and inoculum count					

VI. Results

Contact Time	Contact Time	Organisms	Results			Carrier Population Average CFU/carrier (Average Log_{10})
			Batch No.	Average CFU/carrier	Log Reduction	
No Treatment	20 minutes	*E. coli* spp.	A81625 018CA3			2.5×10^8 (8.4)
		Salmonella spp.	A81625 018CA3			5.5×10^8 (8.74)
		S. enteritidis (PT30)	A81625 018CA3			1.6×10^9 (9.21)
13,000 ppm	20 minutes	*E. coli* spp.	A81625 018CA3	4.57×10^5 (5.66)	2.7	2.5×10^8 (8.4)
		Salmonella spp.	A81625 018CA3	1.3×10^5 (5.12)	3.6	5.5×10^8 (8.74)
		S. enteritidis (PT30)	A81625 018CA3	2.1×10^6 (6.33)	2.9	1.6×10^9 (9.21)
19,000 ppm	20 minutes	*E. coli* spp.	A81625 018CA3	1.00×10^5 (5.01)	3.4	2.5×10^8 (8.4)
		Salmonella spp.	A81625 018CA3	7.6×10^4 (4.88)	3.9	5.5×10^8 (8.74)
		S. enteritidis (PT30)	A81625 018CA3	7.7×10^4 (4.89)	4.3	1.6×10^9 (9.21)
25,000 ppm	20 minutes	*E. coli* spp.	A81625 018CA3	2.5×10^4 (4.4)	4	2.5×10^8 (8.4)
		Salmonella spp.	A81625 018CA3	0 (0)	8.7	5.5×10^8 (8.74)
		S. enteritidis (PT30)	A81625 018CA3	6.0×10^5 (5.78)	3.6	1.6×10^9 (9.21)

Titration of Treatment Solutions-Puma (Lot# A81625018CA3)

Seed Sample	Average $[OCl^-]$ Initial	Average $[OCl^-]$ Final

E. coli O157:H7	13,000 ppm	10,600 ppm
	19,000 ppm	15,500 ppm
	25,000 ppm	21,400 ppm
Salmonella spp.	13,000 ppm	10,000 ppm
	19,000 ppm	16,000 ppm
	25,000 ppm	22,000 ppm
Salmonella PT30	13,000 ppm	10,700 ppm
	19,000 ppm	16,000 ppm
	25,000 ppm	22,000 ppm

VII. CONCLUSIONS

1. The submitted efficacy protocol is **acceptable** for evaluation of the antimicrobial efficacy of sodium hypochlorite for alfalfa seed sanitization.
2. The submitted efficacy data **do not support** the use of Reg. # 5813-100 for alfalfa seed sanitization at a concentration of 13,000 ppm and a contact time of 20 minutes. The treatment did not achieve a 3-log reduction for the test bacteria for the stated contact time.
3. The submitted efficacy data **support** the use of Reg. # 5813-100 for alfalfa seed sanitization at concentrations of 19,000 ppm and 25,000 ppm with a contact time of 20 minutes. The treatment did achieve at least a 3-log reduction for the test bacteria for the stated contact time.

VIII. LABEL RECOMMENDATIONS

1. The sample label language for EPA Reg. # 5813-100 relating to treatment of alfalfa seeds is acceptable.

Note to PM:

1. The sample label for protocol submission for EPA Reg. # 5813-100 was a partial label. The entire label should be submitted for review.
2. The following additional data should be submitted to the agency to further substantiate claims for control of alfalfa seeds for sprouting within a 1-year time frame.
    a. Efficacy testing conducted under GLP as required by 40 CFR 160.
    b. Efficacy testing for three product test lots for each organism.
    c. Certificates of analysis should be provided to substantiate the tested concentration